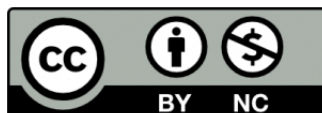




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Analysis of genetic susceptibility to mercury contamination evaluated through molecular biomarkers in at-risk Amazon Amerindian populations

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Abstract

We investigated individual differences in susceptibility to methylmercury (MeHg) contamination and its relationship with polymorphisms of the detoxifying enzyme glutathione S-transferase (GST). In Brazil, some Amerindian tribes from the Amazon region have an increased level of mercury in their hair. Samples of hair and blood were taken from inhabitants of two villages in the Kayabi and Mundurucu Amerindian communities to investigate mercury levels in association with genetic polymorphism of GSTs. Other molecular biological markers were also studied, such as hemoglobin, haptoglobin and glucose 6-phosphate dehydrogenase (G-6-PDH). Higher levels of mercury contamination were found in the Kayabi villagers, who had a null genotype (GSTM1 0/0, also denominated GSTM1 null) frequency of 26%, than in the Mundurucu villagers, for which the null genotype frequency was 0%. Individuals with the GSTM1 null phenotype had higher concentrations of mercury in their hair than individuals with GSTM1+/+ phenotypes ($F = 21.51$, $p < 0.0001$). No association with other markers studied was observed. This study suggests that GSTM1 may be involved in the biotransformation of mercury in humans.

Key words: Amerindian, genetic polymorphism, glutathione S-transferase, mercury.

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Introduction

Mercury, a naturally occurring element that is released from a variety of sources including human activity, is used industrially all over the world because of its diverse properties. In the Brazilian Amazon, mercury contamination of fish can occur as a result of both natural biogeochemical processes and gold-mining activities (Pfeiffer and Lacerda, 1988; Eve *et al.*, 1996; Lechler *et al.*, 2000). Human mercury contamination results primarily from fish consumption because fish not only absorb methylmercury (MeHg) from the water throughout their life but also convert inorganic Hg^{+2} to methylmercury through biomethylation occurring in their body. Fish at the top of a food chain

have the greatest accumulation of methylmercury (Barbosa *et al.*, 1995; Shoeny, 1996; Ashner, 2002). In the Brazilian Amazon, many studies have already been carried out on riverine and Amerindian populations to investigate the health impacts of mercury exposure in different communities (Akagi *et al.*, 1995; Barbosa *et al.*, 1998; Boischio and Henshel, 2000; Santos *et al.*, 2000; Dolbec *et al.*, 2001; Dorea, 2003).

Various factors affect the absorption, distribution, biotransformation, excretion and, consequently, toxicity of methylmercury. It has been pointed out (Doi, 1991) that genetically determined factors play an important role in the differential susceptibility which individuals show to mercury toxicity. It is known reduced glutathione (GSH) and γ -glutamyl transpeptidase (γ -GT) are involved in the disposition and excretion of methylmercury (Strange *et al.*, 2001), and since glucose-6-phosphate dehydrogenase (G-6-PDH) is involved in the production of GSH a defi-

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ciency in G-6-PDH can influence the metabolism and detoxification of mercury (Harris 1980). Methylmercury is exported from liver cells into plasma in complex with GSH because both inorganic and methylmercury have high affinity for reduced sulfhydryl (SH) groups, which bind to biological thiols such as L-cysteine and glutathione to form conjugated complexes. Hepatic methylmercury uptake is increased in the presence of plasma cysteine or GSH and some hemoglobin molecules have extra cysteine moieties or cysteine-SH groups which are more accessible to methylmercury, such compositional and structural differences in hemoglobin being genetically determined and correlated with differences in mercury blood levels (Tsuguyouski *et al.*, 1991).

The glutathione S-transferase (GST) gene family is involved in the detoxification of electrophilic compounds by conjugation, and in higher organisms at least five GSTs gene classes (*alpha*, *mu*, *pi*, *theta* and *zeta*) have been well studied. Each GST class has substrate selectivity depending on the chemical properties of the compounds. The GSH conjugated is more water soluble and therefore immediately excreted via the bile or urine (Armstrong, 1990; Strange *et al.*, 2001). Naganuma *et al.* (1990) and Choi *et al.* (1996) have shown that GSH deficiency is associated with sensitivity to both mercury chloride and methylmercury. In vitro studies have demonstrated conjugation between methylmercury and GSH to form a complex which can interact with GST-*pi*, such binding possibly having a protective function against heavy metals (Almar and Dierickx, 1990). According to Brambila *et al.* (2002) various GST genes are activated in rats exposed to mercury, indicating that individuals with specific genotypes could be better protected against the cytotoxicity of methylmercury. In GST *mu* (GSTM) five *mu* classes occur in tandem (GSTM4-GSTM2-GSTM1-GSTM5-GSTM3) in a cluster on chromosome 1p13.3. Polymorphism of the M1 locus has been widely described. Homozygotes, in which both GSTM1*0 alleles are deleted (GSTM1 null genotype), do not express the GST protein (Xu *et al.*, 1998). With the GST *theta* (GSTT), two genes (GSTT1 and GSTT2) are located on chromosome 22 and separated by about 50 kb. The allele GSTT1*0 is also non-functional (Coggan *et al.*, 1998). The GST *pi* (GSTP) are located on chromosome 11p13 and two different alleles (GSTP1 and GSTP2) have been identified. The wild-type allele differs to the mutant allele by a nucleotide transition on codon 105 of exon 5, which causes in an amino-acid substitution of isoleucine for valine. The mutant that the enzyme (val¹⁰⁵) is 3-fold less effective (Harris *et al.*, 1998).

Some Brazilian Amerindian populations use fish as their main dietary protein source and are thought to be contaminated by mercury. We studied the Kayabi and Munduruku Brazilian Amazonian Amerindian communities for mercury contamination and genetic polymorphism in spe-

cific biological markers associated with mercury metabolism. The connection between polymorphic genes linked to mercury metabolism and the concentration of mercury in hair was also investigated. This study was carried out on loci for hemoglobin, haptoglobin (Hp), glucose-6-phosphate dehydrogenase (G-6-PDH) and the glutathione S-transferase loci *mu* (GSTM1), *pi* (GSTP1) and *theta* (GSTT1).

Material and Methods

Study area and communities

The Kayabi community has an estimated population of 1,000 Amerindians and is situated at 11°37' S and 55°40' W on the banks of the Teles Pires river which has received high emissions of inorganic mercury from the many gold-mining sites which function along its small tributaries. We sampled 65 Kayabi individuals (41% males, 59% females) with an average age of 24.53 ± 15.57 years, Table 1 showing the age classes for our sample population. One blood sample was taken from each individual for analyses of gene polymorphisms. The Munduruku community has an estimated population of 646 Amerindians and is situated at 7°37' S and 57°34' W on the banks of the Teles Pires river, which has received similar mercury contamination. We sampled 117 Munduruku individuals (41% males, 59% females) with an average age of 30.90 ± 15.14 years, Table 1 showing the age classes for our sample population. One blood sample was taken from each individual for analyses of gene polymorphisms.

Both communities are situated in areas of intensive gold prospecting activity and the main diet of the community is fish and starchy root crops, principally cassava (*Manihot ssp.*) flour. This study was approved by the ethics committee of the University of Brasília in accordance with the rules of the Brazilian Ministry of Health. All individuals participating in the study, or their legal guardians, provided informed consent.

Table 1 - Mercury levels in hair from individuals from the Kayabi and Munduruku communities

Community	Age class (years)	Number of individuals per age class (%) ^a	Mean mercury concentration in hair (ppm)
Kayabi	Birth to 20	33 (50)	17.86 ± 9.82
	21 to 40	25 (39)	11.97 ± 6.83
	41 to 60	5 (8)	14.35 ± 6.70
	> 61	2 (5)	15.17 ± 7.13
Munduruku	Birth - 20	34 (29)	4.26 ± 2.16
	21 to 40	56 (48)	3.65 ± 1.88
	41 to 60	20 (17)	3.75 ± 1.67
	> 61	7 (6)	3.72 ± 2.05

^aRounded, may not add up to 100%.

Mercury analysis

For mercury analysis about 10 hairs (10–20 mg) were weighed on an analytical balance (Mettler AE200, Hightstown, NJ) and placed into separate vials which had been previously weighed and labeled. For digestion, 2 mL of 45% NaOH, 1 mL of 1% cysteine and 5 mL of 1% NaCl (all % w/v) was added to each vial and the hair digested on a hot plate at 90–95 °C for 15 min, after which the samples were cooled in crushed ice and any evaporated water replaced. The samples were analyzed for mercury content according to the method of Magos and Clackson (1972) using atomic cold vapor absorption spectrophotometry (CV-AAS) using a model 1255 LDC Analytical Mercury Monitor (LDC, Riviera Beach, FL, USA) having a quantification limit for mercury of 1.5 ppb. The University of Brasilia laboratory is a participant in the Mercury Quality Assurance Program (Hair Mercury-Inter-laboratory Comparison Program, Ottawa, Ontario, Canada).

Gene polymorphism studies

Individual blood samples were screened for hemoglobin variants using 14% (w/v) starch gel electrophoresis from hemolysates and haptoglobin (Hp) gel electrophoresis were carried out with serum samples directly (Ramalho, 1986). For G-6-PD variants analysis, agarose gel electrophoresis were carried out from hemolysates (Harris, 1978). Polymorphism of the GST gene family was determined in DNA extracted from each blood samples using a standard method (GFX Genomic Blood DNA Purification Kit - Amersham Biosciences), followed by PCR amplification using specific primers for GSTM1 (Fryer *et al.*, 1993), GSTT1 (Kemples *et al.*, 1996) and GSTP1 locus 105 (Park *et al.*, 1999), α -globin primers being used as the positive control to check PCR performance. The PCR products were separated using 6% polyacrylamide gel electrophoresis and visualized by silver nitrate staining.

Statistic analyses

The allelic and genotype frequencies of both populations were analysed using the chi-squared test (χ^2) con-

tained in the FREGEM - GENIOC statistical package (Cabello and Krieger 1997). The SAS statistical package version 6 was used to perform analysis of variance (ANOVA) and detect associations between different mercury concentrations in the hair samples, specific genotypes, sex and age.

Results

In our study we found that the mean level of mercury in hair from the Kayabi population was 14.75 ± 1.0 ppm, Table 1 showing the mercury levels for the different age classes. There was no statistically significant differences in hair mercury between the sexes in either community (ANOVA, $p > 0.05$) but, unexpectedly, there were within-population differences as regards age, with individuals up to the age of 20 being more contaminated than individuals in the 21 to > 61 age group (Table 1).

Hemoglobin and G-6-PDH analysis showed no variant that could be associated with variability of hair mercury in either population. The haptoglobin (Hp) allele frequencies (Hp*1 = 0.72 and Hp*2 = 0.28 for the Kayabi; Hp*1 = 0.74 and Hp*2 = 0.26 for the Munduruku) in both communities were in Hardy-Weinberg equilibrium (Table 2) and there was no relationship between the distribution of haptoglobin phenotypes and hair mercury contamination according to age or sex (ANOVA, $F = 1.63$, $p > 0.19$).

Glutathione S-transferase M1 (GSTM1) was selected for study based on the hypothesis that the presence or absence of this allele could be associated with the level of mercury contamination. In the Kayabi population 26% of individuals were homozygous for the non-functional GSTM1 null allele (GSTM1-) but in the Munduruku population all the individuals were monomorphic for the phenotype active GSTM1 (GSTM1+) (Table 3). Individuals with the GSTM1 null phenotype presented (GSTM1-) higher concentrations of hair mercury than GSTM1+ individuals, even considering possible age and sex effects ($F = 21.51$, $p < 0.001$, Table 3). The phenotypic heterogeneity test for GSTM1 polymorphism indicated that Kayabi and Munduruku populations were significantly different ($\chi^2 = 34.26$,

Table 2 - Relationship between haptoglobin (Hp) phenotype and mercury levels in the hair of individuals from the Kayabi and Munduruku Amerindian communities.

Community	Phenotype	Number of individuals per age class (%)	Mean mercury concentration in hair (ppm)	Allele frequency
Kayabi	Hp1-1	34 (52.31)	9.59 ± 0.57	0.72 for Hp*1 ^a
	Hp2-1	25 (38.46)	9.58 ± 0.64	0.28 for Hp*2
	Hp2-2	6 (9.23)	6.48 ± 1.60	
Munduruku	Hp1-1	59 (52.21)	4.26 ± 2.16	0.74 for Hp*1 ^b
	Hp2-1	50 (44.25)	3.65 ± 1.88	0.26 for Hp*2
	Hp2-2	4 (3.54)	3.75 ± 1.67	

^a $\chi^2 = 0.2$ for $p > 0.8$. ^b $\chi^2 = 2.8$ for $0.05 < p < 0.10$.

Table 3 - Phenotypes of GST families with its respective allelic and genotypic frequencies and levels of Hg in the Kayabi and Munduruku communities.

Phenotypes	Mean mercury concentration in hair (ppm)	Allele frequency	Genotypic frequency (%)
Kayabi community			
GSTM1+	8.2 ± 0.44	0.49	74
GSTM1-	14.0 ± 1.36 [#]	0.51	26
GSTT1+	9.49 ± 0.47	0.55	80
GSTT1-	9.29 ± 0.84	0.45	20
GSTP1 ^{Ile/Ile}	14.6 ± 10	GSTP 1* ^(Ile) 57.5	38.3
GSTP1 ^{Val/Val}	15.0 ± 10	GSTP 1* ^(Val) 42.5	23.4
GSTP1 ^{Ile/Val}	15.8 ± 7.9		38.3
Munduruku community			
GSTM1+	3.0 ± 1.2	100	100
GSTM1-	3.24 ± 0.79	0	0
GSTT1+	3.64 ± 0.94	0.50	75
GSTT1-	2.85 ± 0.82	0.50	25
GSTP1 ^{Ile/Ile}	4.26 ± 2.15	GSTP 1* ^(Ile) 67	47.3
GSTP1 ^{Val/Val}	3.64 ± 1.86	GSTP 1* ^(Val) 33	13.4
GSTP1 ^{Ile/Val}	3.75 ± 1.74		39.3

[#]Relationship between high hair mercury and the GSTM1 null phenotype significant by ANOVA at $p < 0.001$.

$p < 0.001$, Table 3. The GSTT1 locus was polymorphic in both populations, with GSTT1 null frequencies of 20% for the Kayabi sample and 25% for the Munduruku sample but there was no relationship between the distribution of the GSTT1 null phenotype and mercury contamination ($F = 0.11$, $p = 0.74$) and the heterogeneity test for distribution of GSTT1 phenotypes between populations was not significant ($\chi^2 = 0.496$, $0.50 < p < 0.70$). The results for the GSTT1 and GSTP1 loci were similar, with the GSTP1 locus presenting polymorphism without any relationship being seen between this allele and the level of mercury in either population ($F = 0.15$, $p = 0.85$, Table 3). The GSTP1 locus showed Hardy-Weinberg equilibrium in both populations ($\chi^2 = 2.79$; $0.05 < p < 0.10$ for the Kayabi and $\chi^2 = 1.4$; $0.30 < p < 0.50$ for the Munduruku).

Discussion

In our study, 62.12% of the Kayabi sample population had a mean hair mercury concentration above the acceptable limit (10 ppm) established by the World Health Organization. Barbosa *et al.* (1995) demonstrated a direct relationship between dietary habits and mercury contamination in different Amazonian populations and showed that gold prospectors have high levels of mercury in their urine due to occupational exposure to inorganic mercury vapor inhaled during amalgam burning.

When released into the environment, divalent mercury (Hg^{+2}) can be converted by sediment bacteria and other organisms into methylmercury (MeHg) which can be bioaccumulated in fish and biomagnified up the food chain. Toxicity of different chemical forms of mercury differs strongly, with MeHg being among the most potent forms (Potter *et al.*, 1975; Richins *et al.*, 1975; Copper, 1983).

Gene flow between the Kayabi and Munduruku communities is very rare because even though they are vicinal cultural differences act as a barrier to marriages between members of these tribes (Rodrigues, *et al.*, 2002). Rodrigues *et al.* (2002) reported also that the Munduruku are more isolated and marriage generally occurs within the tribe, while the Kayabi live in an area that has received intensive migration due to gold prospecting and have consequently become somewhat mixed. The differences observed in the GSTM1 gene frequencies between these two communities suggest that they are heterogeneous. We found that 74% of GSTM1+ phenotype Kayabi sampled had about 8 ppm of mercury in their hair while 26% of GSTM1 null phenotype Kayabi sampled had mercury levels of 14 ppm. On the other hand, all the Munduruku individuals sampled had the GSTM1+ phenotype and low levels of mercury in the hair.

The association between a high frequency of the GSTM1+ allele and lower mercury contamination in the Munduruku sample and the fact that Kayabi GSTM1 null homozygotes had higher levels of mercury than either the GSTM1+ Kayabi or the Munduruku sample suggest that the GSTM1 gene could be involved in mercury metabolism or could be associated with reduced mercury levels. Individual differences in susceptibility to methyl mercury toxicity may be associated with enzymes that exhibit genetic polymorphism. A study by Rodrigues *et al.* (2002) of 308 individuals from the Munduruku showed almost no mercury contamination (a mean of 4.3 ± 0.1 ppm) and our results in the results in table 2 confirm this previous study, showing that the Mundurukus have a lower level of Hg in their hair.

Both communities appear to have received similar mercury exposure because they have the same dietary habits based on fish and (principally cassava) and are located in areas subject to intensive gold prospecting (Rodrigues *et al.*, 2002). Although the Kayabi community has mercury levels in excess of 10 ppm no clinical signals of mercury contamination have been reported (Rodrigues *et al.*, 2002). Medical practitioners who have followed this community recommended they should eat Brazil nuts (Castanha-do-pará), a typical Amazonian nut with a high selenium content which eliminates Hg from the human body (Imura and Naganura, 1985). Fish *per se* are the best sources of essential nutrients as well as selenium and are abundant natural resource for the Amazonian Indians and other river-bank dwellers (Dorea, 2003). However, an other study with mercury in the Amazonian ecosystem have shown high con-

centrations of mercury in fish from the non-polluted freshwater of the Rio Negro which has no history of gold-mining (Barbosa *et al.*, 2003). Mercury biomagnification in piscivorous Rio Negro fish appears to be due to pre-formed methyl mercury occurring naturally in the environment and the food-chain of the fish, demonstrating that the mercury cycle in the Amazonian ecosystem is still not well understood (Barbosa *et al.*, 2003).

Our study indicates that some Kayabi individuals with a specific phenotype could have increased mercury load in their body. Genetic variability endows a species with the ability to adapt to the environment over time, because of which it is important to study the role of genetic factors such as GSTM1 polymorphisms in protecting people from mercury damage.

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